

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Eva Steiness	Confirmation No.:	2651
Serial No.:	10/517,563	Art Unit:	1644
371(c) Date:	July 8, 2005	Examiner:	G. Ewoldt
Customer No.:	21559		
Title:	GLP-1 AND METHODS FOR TREATING DIABETES		

DECLARATION OF KELD FOSGERAU, PH.D. UNDER 37 C.F.R. § 1.132
TRAVERSING GROUNDS OF REJECTION

Under 37 C.F.R. § 1.132 and regarding the rejection of claims 28 and 29 under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement and the rejection of claims 1-22, 24-29, and 36-39 under 35 U.S.C. § 103(a) as being obvious over WO 01/04156 in view of Roach et al., Diabetes Care 22:1258-61, 1999, I declare:

1. I am the Head of Pharmacology at Zealand Pharma A/S.
2. I have been working in the field of Diabetes Pharmacology for 14 years and have been studying incretin biology for over 5 years. A copy of my *curriculum vitae* is enclosed.
3. I have read and understood the Office action dated June 18, 2010.
4. I disagree with the contention that a scientist or physician of ordinary skill would not have been in possession of "insulin analogs recognized as antidiabetic drugs" at the time the application was filed. As evidenced by Vajo et al., Endocrine Rev 22:706-17, 2001, a variety of such analogs were known in art. I therefore believe a skilled scientist, working in the field of diabetes research at the time the application was filed, would either know the identity of such analogs or would readily be able to determine their identity.

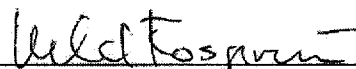
5. I also disagree that the results provided in the application cannot be generalized to additional GLP-1 agonists. To the end, I have performed experiments similar those described in the application. In these experiments, male *db/db* mice were obtained at an age of 5-6 weeks. As shown in accompanying Figure 1, the mice were divided into seven groups. Groups 1-3 were administered a vehicle for 50 days followed by administration of either the vehicle, Byetta, or Victoza, respectively, for 43 additional days. Groups 4 and 6 were administered Byetta or Victoza, respectively, for the entire 93 days, and Groups 5 and 7 were administered Byetta or Victoza, respectively, for 50 days, followed by administration of a vehicle for 43 additional days. Oral Glucose Tolerance Tests (OGTT) as well as fasting blood glucose measurements were performed on the mice during the course of the experiment.

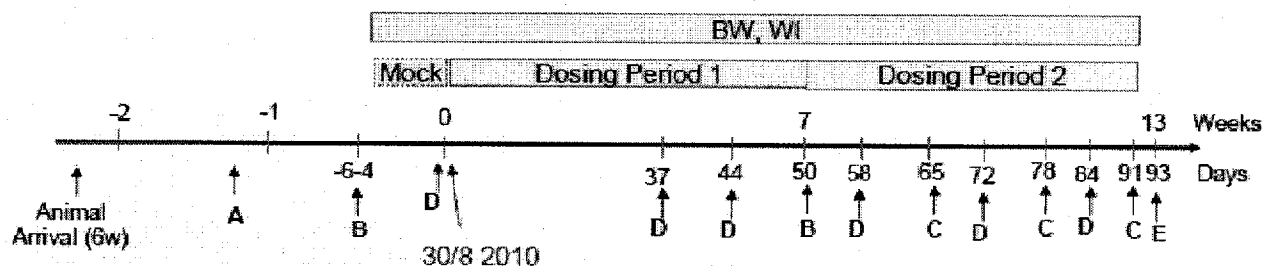
5. As shown in Figure 2, OGTT results indicate that mice administered either Byetta or Victoza for 50 days followed by administration of vehicle for 43 days exhibited improved glucose responses during the 43-day period, as compared to the mice receiving the vehicle for the entire 93 days. As shown in Figure 3, similar improvements were observed in the fasting blood glucose measurements.

6. Based on these data, I conclude the experimental results involving des Pro³⁶-exendin-4(1-39)-Lys₆-NH₂ ("COMPOUND 1") described in the application can be generalized to other GLP-1 agonists.

7. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

17/12 2010
Date


Keld Fosgerau, Ph.D.



Analysis

- A. Baseline (n=150): BG, HbA1c, C-peptide
- B. Stratification (n=130) according to OGTT
- C. OGTT (BG: t = (-) 0, 15, 30, 60, 120, 240 min)
- D. Fasting (8hr) BG
- E. Termination: BG, HbA1c, C-peptide, pancreas weight and insulin content.

Groups (n=13/ group)	Substance Period 1	Substance Period 2	Route	Dose (nmol/kg/day)
Group 1	Vehicle	Vehicle	SC once daily	-
Group 2	Vehicle	Byetta		0+100
Group 3	Vehicle	Victoza		0+100
Group 4	Byetta	Byetta		100+100
Group 5	Byetta	Vehicle		100+0
Group 6	Victoza	Victoza		100+100
Group 7	Victoza	Vehicle		100+0

Figure 1

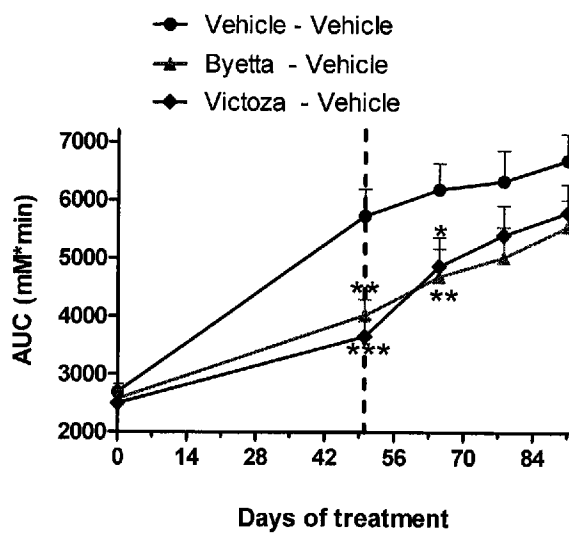
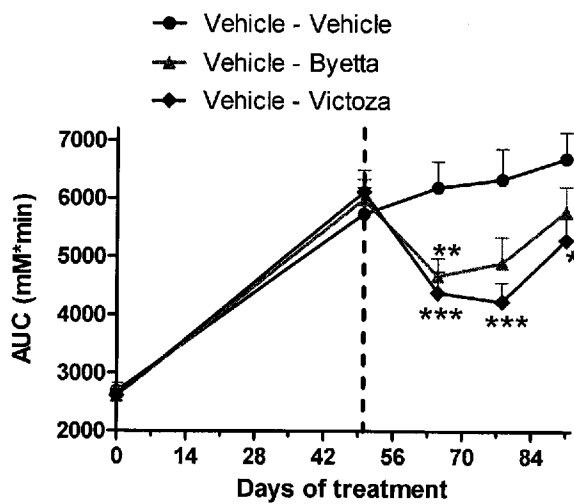
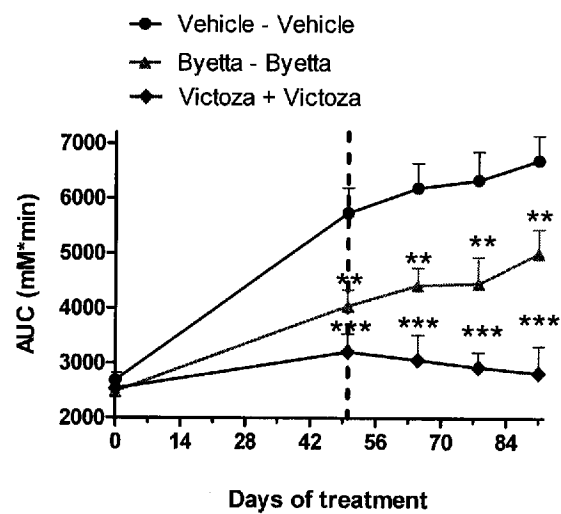


Figure 2

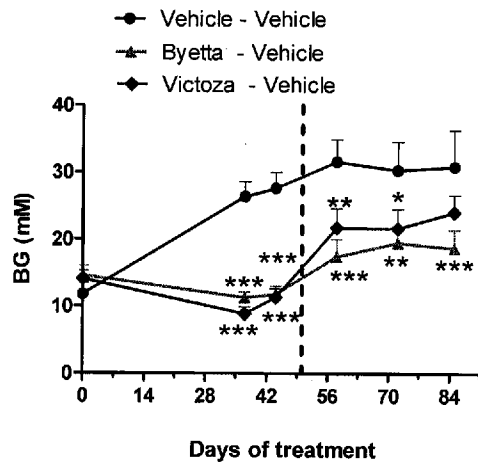
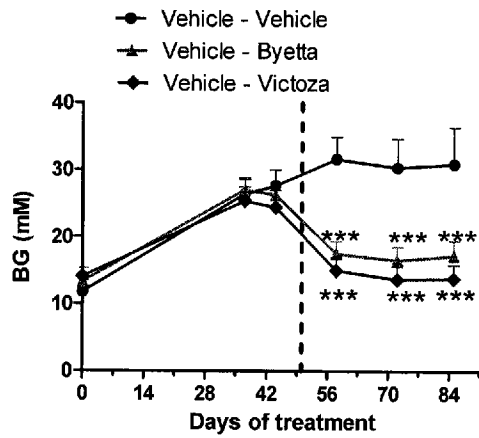
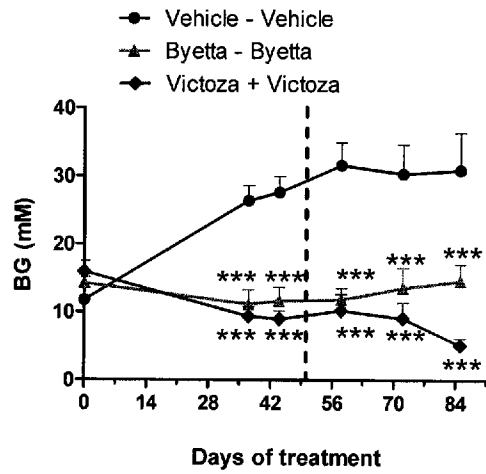


Figure 3

Curriculum Vitae (CV)

1. PERSONAL DATA

Name	Keld Fosgerau
Current position	Head of Pharmacology
Date of birth	09-02-1969
Nationality	Danish

2. EDUCATION

2000:	PhD University of Copenhagen, industrial PhD fellowship program
1995:	Master in biological chemistry Danish Technical University

3. FURTHER TRAINING

- Your personal leadership (DIEU)
- Management course for academics (DIEU)
- Introduction to management (DIEU)
- Project management foundation program 2 (DIEU)
- Project management foundation program 1 (DIEU)
- Introduction to project management (NN)
- Technology management and creation course (HHK)
- Patent course for biologists (NN)
- Patent course (NN)
- Basic radiation safety in the laboratory (USC, LA, USA)
- Pain treatment in laboratory animals (DVAU)
- Advanced statistics for academics (NN)
- Basic statistics for academics (NN)
- And other...

4. PROFESSIONAL CAREER

2008-2010: **Neurokey A/S**
Director of Preclinical Development

With my position at Neurokey I have the great opportunity of learning about the disciplin of preclinical development, and to get closer to the clinical trials and the actual issuing of a pharmaceutical. Neurokey is a small start-up pharmaceutical company, founded in 2007, where I am responsible for toxicology and pharmacology activities. A large part of the job

has been focused on the preclinical development of the previous lead compound, and I have been responsible for identification, evaluation and collaboration with external pre-clinical international CRO's and consultants. With support from 1 principal scientist I have performed a complex toxicology program and developed an advanced model for proof-of-concept. Working as preclinical project manager I have collaborated closely with our clinical and regulatory responsible to plan the optimal progress of the lead program to Phase II. In addition to the work on the previous lead compound I have been key in evaluation of scientific aspects of several other project opportunities for future development in the field of critical care and in particular neuroprotection.

2006-2008: Rheoscience A/S
Manager, Senior Scientist

Moving to biotech and Rheoscience gave me the opportunity of gaining more experience with both project and line management. Accordingly the position at Rheoscience was multi-faceted and I was involved both in the Discovery and CRO function of the company. Discovery roles: Evaluation of novel project ideas. Project manager for a full discovery project in the field of diabetes involving about 15 scientists from both in house resources as well as an international CRO (Aurigene, India). The work included set up of screening plan and progress of NCE from drawing board to the in vivo phase. Also, I was responsible in the coordination of Rheoscience participation in an EU FP7 project on exercise and obesity (Exgenesis) collaborating with several international partners. CRO roles: daily manager of 25 people including 6 scientists at PhD level, and I was a study director and key account manager for several central costumers providing advanced pharmacology studies and consultancy in the area of diabetes and obesity.

2000-2006: Novo Nordisk A/S
Research Scientist

Going from the university to the industry I enjoyed the speed and possibility of working with applied research. At Novo Nordisk my focus was to gain specialist level knowledge within diabetes and obesity pharmacology. I coordinated pharmacology activities in early stage discovery projects and participated in identification of novel drug targets. I was appointed as modulator of cross-functional innovation forum for scientists, which gave experience in leading larger scientific group discussion. I became experienced in collection, analysis, and presentation of in-house and ex-house research results with special focus on advanced in vivo methods for the investigation of metabolic regulation.

International experience

2003 Visiting scientist at Garvan Institute of Medical Research,

Sydney, Australia (3 months): *Bromo-palmitate tracer technique for determination of FFA utilization in rats.*

1999 PhD scholar at University of Southern California, USA (10 months): *Euglycemic-hyperinsulinemic clamp technique in dogs*

5. LECTURING (PRE- AND POST-GRADUATE)

- 4 Master students
- 2 PhD students
- Challenge of 1 PhD defend

6. PUBLICATIONS

14 issued publications, hereof 8 as first author.
Appendix 1: Publications

7. LECTURES AND POSTERS AT SCIENTIFIC MEETINGS

>25 posters at international meetings
Appendix 2: Abstract and Proceedings

8. MEMBERSHIP OF SCIENTIFIC / OCCUPATIONAL SOCIETIES

None

9. EDITORIAL ACTIVITIES

None

10. REFEREE FOR SCIENTIFIC JOURNALS

Occasional single assignments

11. MISCELLANEOUS

License to perform animal experiments (Dyreforsøgstilsynet)
License to work with isotopes (Dansk institut for stråling)

12. CIVIC ACTIVITIES

Date of last revision

Curriculum Vitae (CV)

Keld Fosgerau
09-02-1969

ZEALAND

P H A R M A

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Date of present revision 01-11-2010

Authentication of the present CV

APPENDIX**Appendix 1. Publications**

1. Fosgerau K, Weber UJ, Gotfredsen JW, Jayatissa M, Buus C, Kristensen NB, Vestergaard M, Teschendorf P, Schneider A, Hansen P, Raunsø J, Køber L, Torp-Pedersen C, Videbaek C (2010) Drug-induced mild therapeutic hypothermia obtained by administration of a transient receptor potential vanilloid type 1 agonist - *BMC Cardiovasc. Dis.* 10:51.
2. Fosgerau K, Ristagno G, Jayatissa M, Axelsen M, Gotfredsen JW, Weber UJ, Køber L, Torp-Pedersen C, Videbaek C (2010) Increased susceptibility to cardiovascular effects of dihydrocapsaicin in resuscitated rats. *BMC Cardiovasc. Dis.* 10:39.
3. Madsen AN, Hansen G, Paulsen J, Lykkegaard K, Hansen HS, Levin, BE, Larsen PJ, Knudsen LB, Fosgerau K, Vrang N (2010) Long-term characterization of the diet-induced obese and diet resistant rat model: A polygenetic rat model mimicking the human obesity syndrome. *J. Endocrinol.* 206, 287-96.
4. Fosgerau K, Hansen T, Galle P, Albrechtsen A, Rieper CL, Pedersen BK, Larsen LK, Thomsen AR, Pedersen O, Hansen MB, Steensberg A (2010) Interleukin-6 autoantibodies are involved in the pathogenesis of a subset of Type 2 diabetes *J. Endocrinol* 204, 1-9.
5. Suh SW, Bergher JP, Anderson CM, Treadway JL, Fosgerau K, Swanson RA (2007) Astrocyte glycogen sustains neuronal activity during hypoglycemia: studies with the glycogen phosphorylase inhibitor CP-316,819. *J. Pharmacol. Exp. Ther.* 321 (1): 45-50.
6. Sørensen H, Winsell MS, Brand CL, Fosgerau K, Gelling RW, Nishimura E, Ahrén B (2006) Glucagon receptor knockout mice display increased insulin sensitivity and improved beta cell function. *Diabetes* 55 (12): 3463-3469.
7. Sørensen H, Brand CL, Neschen S, Holst JJ, Fosgerau K, Nishimura E, Shulman GI (2006) Immuno-neutralization of endogenous glucagon reduces hepatic glucose output and improves long-term glycemic control in diabetic ob/ob mice. *Diabetes* 55 (10): 2843-2848.
8. Fosgerau K, Fiedelius C, Pedersen KE, Kristensen JB, Daugaard JR, Iglesias MA, Kraegen EW, Furler SM (2006) Oral administration of glucose promotes intracellular partitioning of fatty acid toward storage in white but not in red muscle. *J. Endocrinol.* 190: 651-658.
9. Sickmann HM, Schousboe A, Fosgerau K, Waagepetersen HS (2005) Compartmentation of lactate originating from lactate and glucose in cultured astrocytes. *Neurochem. Res.* 30 (10): 1295-1304.
10. Brown AM, Sickmann HM, Fosgerau K, Lund TL, Schousboe A, Waagepetersen HS and Ransom BR (2005) Astrocyte glycogen metabolism is required for neural activity during aglycemia or intense stimulation in mouse white matter. *J. Neurosci. Res.* 79: 74-80.

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11. Fosgerau K, Breinholt J, McCormack JG, Westergaard N (2002) Evidence against glycogen cycling of gluconeogenic substrates in various liver preparations, *J. Biol. Chem.* 270 (37) 28648-28655.
 12. Fosgerau K, Mittelman SD, Dea MK, Sunehag A, Lundgren K, Westergaard N, Bergman RN (2001) Lack of hepatic 'interregulation' during inhibition of glycogenolysis in a canine model. *Am. J. Physiol. Endocrinol. Metab.* 281, E375-E383
 13. Fosgerau K, Westergaard N, Quistorff B, Grunnet N, Kristiansen M, Lundgren K (2000) Kinetic and functional characterization of 1,4-dideoxy-1,4- imino-D-arabinitol: A potent inhibitor of glycogen phosphorylase with anti-hyperglycemic effect in ob/ob mice. *Arch. Biochem. Biophys.* 380 (2) 274-284.
 14. Fosgerau K (1999) Effects of 1,4-dideoxy-1,4-imino-D-arabinitol on glycogen metabolism in various biological systems *in vitro* and *in vivo*. PhD thesis.

2 issued patents.

Sangamesh B, Fosgerau K, Vrang N. Azine compounds as glucokinase activators. Patent No. WO2009/083553 A1.

McKay P, Valcarce-Lopez C, Bödvarsdóttir T, Fosgerau K, Larsen MO, Arkhammer P, Wahl P. Use of liver-selective glucokinase activators. Patent No. WO2005/123132.

Appendix 2. Lectures and Posters

- Chung SP; Park J; Weng Y; **Fosgerau K**; Wu X; Sun S; Weil MH; Tang W (2010) The Effect of Dihydrocapsaicin to Induce Mild Hypothermia in Anesthetized Pigs Circulation, 122: A128.
- Song F; **Fosgerau F**; Yu T; Weng Y; Chung S; Sun S; Weil MH; Tang W (2010) Trpv1 Agonist Rinvanil Induces Pharmacological Hypothermia and Improves the Outcome of CPR in a Rat VF Model Circulation. 122, A165
- Madsen AN, Hansen G, Vrang, N, Tang-Christensen M, Larsen PJ, **Fosgerau K** (2008) Voluntary exercise does not modify body weight set-point in diet-induced obesity prone rats NAASO, US.
- G, Madsen AN, Jørgensen J, Vrang, N, Tang-Christensen M, Larsen PJ, **Fosgerau K** (2008) Voluntary exercise modifies body weight set-point in diet-induced obesity prone rats SSIB, US.
- Bödvarsdóttir TB, Wahl P, Santhosh KC, Polsetti DR, Guzel M, **Fosgerau K**, Larsen MO, Halberg IB, Selmer J, Andrews RC, Mjalli AMM, Valcarce C (2008) TTP355 A Glucokinase Activator – accepted oral presentation ADA.
- Sundell CL, **Fosgerau K**, Chen X, Dodd G, Tang-Christensen M, Kunsch C (2008) AGI-1067, a Novel Antioxidant and Anti-Inflammatory Agent, Improves Insulin Sensitivity in a Rat Model of Diet-Induced Obesity – accepted oral presentation ADA.
- Hansen G, Vrang, N, Tang-Christensen M, Larsen PJ, **Fosgerau K** (2008) Exercise modifies body weight set-point in diet-induced obesity prone rats Keystone Symposia, Keystone, US.
- Larsen J, Lyderik P, Rubach I, Jakobsen JM, Petersen L, **Fosgerau K** (2007) Effect of isoprenaline on heart rate in conscious rats, The 37th ScandLAS.
- Rakipovski G, Jensen MW, Pedersen KE, Andersen H, Breinholt J, **Fosgerau K** (2007) Inhibition of glycogen phosphorylase attenuates hepatic glucose disposal in normal and insulin resistant rats. Diabetologia Suppl. 1: S256.
- A Steensberg, **Fosgerau K**, Galle P, Hansen T, Albrechtsen A, Rieper CL, Pedersen BK, Larsen LK, Thomsen AR, Pedersen O, Hansen MB, (2007) Increased levels of auto-reactive Interleukin-6 antibodies in patients with Type 2 diabetes. Diabetes Suppl. 1:
- **Fosgerau K**, Galle P, Hansen T, Albrechtsen A, Pedersen BK, Larsen LK, Thomsen AR, Pedersen O, Hansen MB, Adam Steensberg (2007) Interleukin-6 auto-reactive antibodies are associated with Type 2 diabetes in humans and induce obesity in mice, Keystone Symposia, Keystone, US.
- Fledelius C, **Fosgerau K**, Vinterby A, Pedersen KE, Wulff EM, de Jong JC, Tornqvist HE, Jacobsen P (2006) The effect of two distinct anti-lipolytic strategies on FFA metabolism in rats in vivo, IDF, Cape Town.

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- **Fosgerau K**, Fledelius C, Pedersen KE, Andersen H, Jeppesen CB, Nishimura E, Andersen HS, Kampen GT (2006) Activation of the nicotinic acid receptor HM74a *in vivo* is associated with rebound effects, Keystone Symposia, Taos New Mexico, US.
 - **Fosgerau K**, Fledelius C, Pedersen KE, Kristensen JB, Petersen H, Kraegen EW, Furler SM (2005) Glucose administration promotes intracellular partitioning of FFA towards storage in liver and white muscle of fasted rats. *Diabetes* 54 Suppl. 1: A354.
 - Waagepetersen HS, Sickmann HM, **Fosgerau K**, Schousboe A (2005) Role of glycogen in neurotransmission. *J Neurochem* 94 Suppl. 1: 65.
 - Pedersen KE, Petersen H, Kristensen JB, Fledelius C, **Fosgerau K** (2005) Permanent catheter technique in conscious rats used for investigation of free fatty acid utilization in liver, muscle and heart. The 35th ScandLAS, Uppsala, Sweden.
 - **Fosgerau K**, Petersen H, Pedersen KE, Kristensen JB, Fledelius C (2005) Acute effect of acipimox and glucose on fatty acid metabolism in rat liver and heart. Keystone Symposia, Keystone Colorado, US.
 - Sørensen H., Brand CL, **Fosgerau K**, Gelling R; Nishimura E, Åhrén B (2004) Glucagon receptor knockout mice display increased insulin sensitivity and improved beta cell function. *Diabetologia*, 47, A91.
 - Pedersen KE, Wulff EM, Kryger S, Hasselager E, Petersen H, **Fosgerau K** (2004) Refinement of method for cannulation of vena jugularis, vena portae and aorta in conscious unrestrained rats. The 34th ScanLAS, Tallinn, Estonia (abstract).
 - Rakipovski G, Petersen H, Pedersen KE, **Fosgerau K** (2004) Inhibition of glycogen phosphorylase attenuates glycolysis in high fat fed rats. Keystone Symposia, Banff, Canada.
 - Rytved KA, Næsse TB, Christensen TT, **Fosgerau K**, Andersen MP, Kiehr B, Sjögren I & Nyborg NCB (2003) Inhibition of glycogen phosphorylase eliminates arrhythmia and reduces infarct size in the rabbit heart. *PhysPharm*, P03-12.
 - Nyborg NCB, Kiehr B, **Fosgerau K**, Rytved KA (2003) Inhibition of myocardial glycogen phosphorylase reduces infarct size following a global ischemic period in anesthetized rabbits *Diabetes* 52, A163.
 - **Fosgerau K**, Nyborg NCB, Sjögren I, Rytved KA (2003) Inhibition of glycogen phosphorylase prevents arrhythmia following a global ischemic period in the perfused rabbit heart. *Diabetes* 52, A154.
 - **Fosgerau K**, Pedersen KE, Petersen H, Bentzen B, and Sjögren I (2003). 24h-infusion of unbound oleic acid via the portal vein causes liver damage. Keystone Symposia, Keystone Colorado, US.

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- Fledelius, C, **Fosgerau K**, Vinterby A, Heding K, Petersen H, Damgaard J, Trolle-Christensen A, Benthem B, and Wassermann K (2002) Effects of acipimox on energy expenditure and substrate oxidation in the fasted rat. Keystone Symposia, Keystone Colorado, US.
 - **Fosgerau K**, Breinholt J, Pedersen KE Westergaard N (2001) Inhibition of glycogenolysis has no effect on gluconeogenesis and glycogen synthesis in fasted rats. Diabetologia, 44, A162.
 - **Fosgerau K**, Mittelman SD, Dea MK, Sunehag A, Lundgren K, Westergaard N, Bergman RN (2000) Inhibition of glycogenolysis without compensatory increases of gluconeogenesis in a canine model. Diabetologia, 43, A153.
 - Andersen B, **Fosgerau K**, Kristiansen M, Lundgren K, Westergaard N (1999) Inhibition of glycogen phosphorylase and glycogenolysis in primary rat hepatocytes by 1,4-dideoxy-1,4-imino-D-arabinitol. Diabetes, 48 Suppl. (1) 1-A-550.
 - **Fosgerau K**, Kristensen M, Westergaard N, Justesen P, Mortensen EG and Lundgren K (1997) 1,4-dideoxy-1,4-imino-D-arabinitol represent a novel class of potent glycogen phosphorylase inhibitors. The 47th Harden Conference, Cirencester, UK.

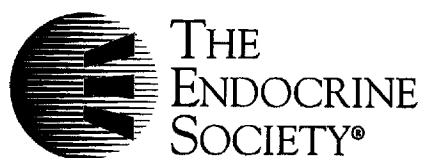
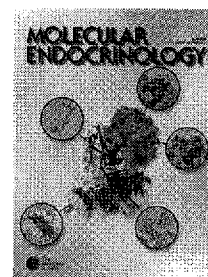
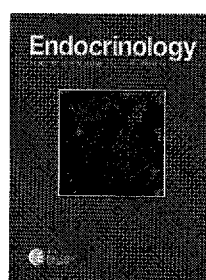
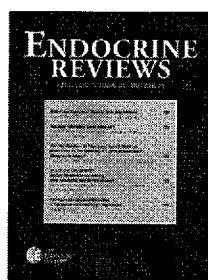
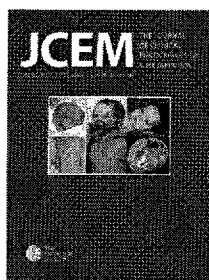
ENDOCRINE REVIEWS

Recombinant DNA Technology in the Treatment of Diabetes: Insulin Analogs

Zoltan Vajo, Janet Fawcett and William C. Duckworth

Endocr. Rev. 2001 22: 706-717, doi: 10.1210/er.22.5.706

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Recombinant DNA Technology in the Treatment of Diabetes: Insulin Analogs

ZOLTAN VAJO, JANET FAWCETT, AND WILLIAM C. DUCKWORTH

Section of Endocrinology, VA Medical Center, Phoenix, Arizona 85012

After more than half a century of treating diabetics with animal insulins, recombinant DNA technologies and advanced protein chemistry made human insulin preparations available in the early 1980s. As the next step, over the last decade, insulin analogs were constructed by changing the structure of the native protein with the goal of improving the therapeutic properties of it, because the pharmacokinetic characteristics of rapid-, intermediate-, and long-acting preparations of human insulin make it almost impossible to achieve sustained normoglycemia. The first clinically available insulin analog, lispro, confirmed the hopes by showing that improved glycemic control can be achieved without an increase in hypoglycemic events. Two new insulin analogs, insulin glargine and insulin aspart, have recently been approved for clinical use in

the United States, and several other analogs are being intensively tested. Thus, it appears that a rapid acceleration of basic and clinical research in this arena will be seen, which will have direct significance to both patients and their physicians. The introduction of new short-acting analogs and the development of the first truly long-acting analogs and the development of analogs with increased stability, less variability, and perhaps selective action, will help to develop more individualized treatment strategies targeted to specific patient characteristics and to achieve further improvements in glycemic control. Data on the currently available and tested analogs, as well as data on those currently being developed, are reviewed. (*Endocrine Reviews* 22: 706-717, 2001)

- I. Introduction
- II. Background
- III. Why Do We Need Analogs?
- IV. Short-Acting Insulin Analogs
 - A. Insulin Asp(B10)
 - B. Insulin lispro (Eli Lilly & Co., Indianapolis, IN)
 - C. Insulin aspart [NovoLog (Novo Nordisk, Princeton, NJ)]
- V. Long-Acting Insulin Analogs
 - A. NovoSol Basal (Novo Nordisk)
 - B. Insulin glargine [HOE 901, LANTUS (Aventis Pharmaceuticals, Parsippany, NJ)]
 - C. Fatty acid-acylated insulins
- VI. Remaining Tasks
 - A. Selective action
 - B. Increased stability
 - C. Less variability
 - D. Ultrarapid onset
 - E. Ultralong activity
 - F. Benefit without metabolic activity?
- VII. Conclusions

I. Introduction

FOR THE PURPOSES of this review, we define insulin analogs as molecules brought about by modifying the structure of the human insulin molecule, which results in altered physicochemical, biological, and pharmacodynamic properties. The first such agent, insulin lispro, has been available to the clinician since mid-1996. No new analogs became available for clinical use for the next 4 yr. However, at the

time of the preparation of this manuscript, two new insulin analogs, insulin glargine and insulin aspart, have recently been approved for clinical use in the United States, and several other analogs are being intensively tested. Thus, it appears that a rapid acceleration of basic and clinical research will be seen in this arena, which will have direct significance to both patients and their physicians.

II. Background

The insulin receptor is a tyrosine kinase that undergoes activation upon insulin binding, leading to the tyrosine phosphorylation of a specific collection of intracellular proteins (1). The IGF-I receptor and the insulin receptor exhibit substantial structural homology to each other, and both ligands have a measurable affinity to the other's receptor. Structure-function studies have shown that the amino acids of the insulin molecule essential for binding to the insulin receptor are A1Gly (glycine at position 1 of the A chain of insulin; Fig. 1), A2Ile, A3Val, A19Tyr, B6Leu, B12Val, B23Gly, B24Phe, and B25Phe, whereas alterations in the B10 and B26-30 regions of the human insulin molecule alter its affinity to the IGF-I receptor (2, 3). Based on this knowledge, it is possible to design analogs of human insulin that preserve receptor binding but show differences in other properties.

III. Why Do We Need Analogs?

In nondiabetic individuals, ingestion of food results in a relatively rapid rise of serum insulin concentration to a maximum after 30-45 min, followed by a decline to basal levels after 2-3 h. The pharmacokinetic characteristics of the currently available rapid-, intermediate-, and long-acting prep-

Abbreviations: CSII, Continuous sc insulin infusion; NPH, neutral protamine Hagedorn.

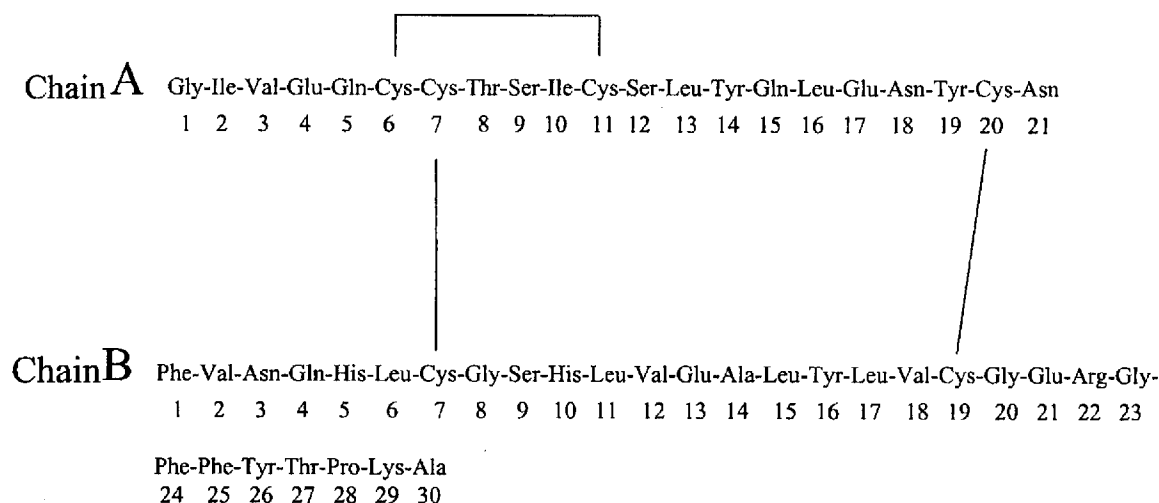


FIG. 1. The amino acid sequence of human insulin. B10 was replaced by Asp in Asp(B10), B28–29 was reversed in lispro, B28 was replaced with Asp in aspart, A21 was replaced with Gly, and Arg was added to B31–32 in HOE 901. Alanine, Ala; arginine, Arg; asparagine, Asn; aspartic acid, Asp; cysteine, Cys; glutamic acid, Glu; glutamine, Gln; glycine, Gly; histidine, His; isoleucine, Ile; leucine, Leu; lysine, Lys; methionine, Met; phenylalanine, Phe; proline, Pro; serine, Ser; threonine, Thr; tryptophan, Trp; tyrosine, Tyr; valine, Val.

arations of human insulin make it almost impossible to achieve sustained normoglycemia. The onset of action of sc-injected regular human insulin is too slow and the duration of its action too long to mimic the insulin secretion pattern of a healthy individual during ingestion of a carbohydrate-containing meal (4). As a result, early postprandial hyperglycemia followed by an increased risk for hypoglycemia before the next meal are present. Similarly, the available intermediate/long-acting human insulin preparations are unable to provide a stable, continuous baseline insulin level. Instead, they cause peak serum insulin levels at 3–4 h after sc injection and show considerable inter- and intra-subject variations in their bioavailability. The Diabetes Control and Complications Trial confirmed the link between glycemic control and the complications of diabetes (5). Therefore, to achieve improved glucose control, the need for new insulin preparations with a faster onset and shorter duration of action and for long-acting preparations with a more flat time-action profile and less variable bioavailability became apparent in the late 1980s and early 1990s (6). However, until recently, improvements in insulin formulations were seriously limited; advances were only achieved in insulin purity, species, and characteristics of the retarding agent. The availability of molecular genetic techniques opened new windows for creating insulin analogs by changing the structure of the native protein, improving its therapeutic properties.

In addition to its glucose-lowering effect, insulin is the most potent physiological anabolic agent known to date (7). It promotes the synthesis and storage of lipids, proteins, and carbohydrates and prevents their degradation and release back to the circulation. Despite years of intensive investigation, we are still left with considerable uncertainty regarding the precise intracellular events that mediate the action of this hormone. One confounding factor has been the variety of actions of insulin, which depend on the cell type, time of exposure, and the presence or absence of other hormones (8). Another is the fact that insulin can act as a growth factor for

cultured cells and shares many of the mitogenic signaling pathways elicited by other growth factors. However, the metabolic effects of insulin are unique and cannot be reproduced by other cellular stimuli (7, 9). Taken together, these findings indicate that signaling mechanisms that respond only to insulin exist, and they allow for the specialized effects of insulin on metabolism. Designing and studying insulin analogs has helped, and without any doubt will help, our understanding of the complex processes insulin is associated with, and creating analogs selective to one or another of insulin's actions might well be of clinical significance.

IV. Short-Acting Insulin Analogs

In addition to the above, regular insulin has more disadvantages. Because of its relatively slow onset of action, regular insulin is optimally administered 30–60 min before meals. Due to the inconvenience and difficulties with predicting the time of the meal, most patients do not follow this advice, even when adequately instructed (10, 11). Therefore, short-acting analogs that could be injected immediately before meals would improve compliance with treatment recommendations and the patients' overall satisfaction with the regimen. Patients consider the opportunity to inject insulin immediately before the meal an advantage, because it can increase flexibility and freedom in daily activities (12). This cannot, however, be achieved by human insulin, because above physiological concentrations, such as those present in the injectable preparations, native human insulin forms dimers and hexamers, which inhibit its rapid absorption from the injection site (13). Therefore, a possible approach to facilitate absorption and to achieve rapid action is to develop analogs with a decreased tendency to self-associate. This can be accomplished by changing the amino acid sequence of human insulin. Because of faster absorption, a substantial reduction in the postprandial glucose excursion is expected

TABLE 1. Structure, characteristics, and status of insulin analogs

Analog	Structure	Characteristics	Status
NovoSol	Arg(B27)Gly(A21)	Long action, low bioavailability	Trials discontinued
Basal	Thr(B30)		
Asp(B10)	Asp(B10)	Short acting, rapidly absorbed, increased metabolic potency	Trials discontinued
Lispro	Lys(B28)Pro(B29)	Short acting, rapidly absorbed	Available since 1996
Aspart	Asp(B28)	Short acting, rapidly absorbed	Approved, but not marketed yet
HOE 901	Gly(A21)Arg(B31)Arg(B32)	Long acting, peakless action, low rates of hypoglycemia	Available for clinical use
WW99-S32	N(ϵ)-palmitoyl Lys(B29)	Long action, less variation, highly reproducible pharmacokinetic profile	Preclinical and early clinical trials
NN304 (detemir)	LysB29-tetradecanoyl, des(B30)	Long-acting, peakless action, less variation	Preclinical and early clinical trials

with such analogs, and the more rapid decline in the serum concentration of the analog should result in a reduced risk of late hypoglycemia compared with regular human insulin (14).

A. Insulin Asp(B10)

Elucidating the genetic basis for a case of familial hyperproinsulinemia (it involves a single-point mutation in the proinsulin gene resulting in the substitution of aspartic acid for the naturally occurring histidine for residue 10 of the B chain of insulin) led to the development of insulin Asp(B10), one of the first insulin analogs proposed for clinical use (15) (Fig. 1 and Table 1). Insulin Asp(B10) is absorbed twice as rapidly as regular insulin and offers potential therapeutic benefits (16). However, studies with Asp(B10) pointed out that a potential problem with altering the amino acid sequence of human insulin is that it can change the three-dimensional structure of the molecule in a way that results in altered interaction with the insulin receptor and the IGF-I receptor. This analog has been demonstrated to have an increased affinity both for the insulin and for the IGF-I receptor, a decreased rate of dissociation from the insulin receptor, as well as prolonged cellular processing (17–19). This results in a much greater metabolic effect compared with human insulin, which would be a potential therapeutic advantage. In addition to carbohydrate metabolism, insulin Asp(B10) been shown to have an increased effect on lipogenesis as well (20). Unfortunately, the above characteristics also lead to increased mitogenic activity in several cell lines, and as a result, suprapharmacological doses of Asp(B10) cause a dose-dependent increase in the incidence of adenocarcinomas in laboratory animals (20–22). Further clinical studies with this analog were therefore halted. However, realizing the enormous potential implications brought about by modifying the human insulin molecule encouraged researchers to continue developing new insulin analogs. At the same time, the significantly different clinical properties of Asp(B10) also boosted a new area of insulin research investigating the biochemical processes other than carbohydrate metabolism in which insulin participates and the processes through which the insulin molecule goes after receptor binding. Research on the analog Asp(B10) has provided useful information in the context of insulin action.

In contrast to human insulin, Asp(B10) induces a prolonged phosphorylation state of the 95-kDa receptor β -

subunit and of the insulin receptor substrates 1/2 and Shc (23). In addition, an increased and prolonged tyrosine phosphorylation of a yet unidentified 60-kDa protein has been observed with Asp(B10). Asp(B10) also shows increased [3 H]thymidine incorporation into DNA compared with regular insulin (23).

It appears that the increased mitogenic activity of Asp(B10) could result from at least two mechanisms, increased IGF-I receptor affinity and decreased dissociation from the insulin receptor. This could be of clinical significance, because when a new analog is developed, it is possible that one or both of these characteristics will be altered. The lesson learned from insulin Asp(B10) was that assessing the molecular pharmacological properties, such as insulin and IGF-I receptor binding and metabolic and mitogenic potency, is of clinical importance in the evaluation of newly developed insulin analogs.

B. Insulin lispro (Eli Lilly & Co., Indianapolis, IN)

The B26–30 region of the insulin molecule is not critical in binding to the insulin receptor. However, it is clearly important in mediating the formation of insulin dimers (24). Therefore, structural modifications of the molecule at these positions would be expected to generate insulin analogs with minimal tendency for self-association but unaltered affinity to the insulin receptor compared with regular human insulin (3).

The first genetically engineered rapid-acting insulin analog to become available for the clinician was insulin lispro, which was approved for clinical use in Europe in April of 1996 and in the United States in June of 1996. In insulin lispro, the normal sequence of proline at position 28 of the B chain and lysine at position 29 is reversed (LysB28,ProB29) (Fig. 1 and Table 1). This reversal causes a decreased tendency for self-association, and as a result, faster absorption, higher peak serum levels, and shorter duration of action can be observed with insulin lispro compared with regular insulin (25). Importantly, as discussed above, the amino acid sequence changes in lispro do not affect its receptor-binding domain. Therefore, the affinity to the insulin receptor of insulin lispro is similar to that of regular insulin. Although lispro's affinity for the IGF-I receptor is slightly higher, it is not enough to cause a difference in its cell growth-stimulating activity compared with regular insulin (26, 27). Also, in the case of lispro, growth-promoting activity in human mam-

mary epithelial cells has been found to be correlated more with dissociation kinetics from the insulin receptor, which were shown to be identical with those of human insulin (3). Insulin lispro was also found to have a low mitogenic potency when studied using a human osteosarcoma cell line (20), and in contrast to Asp(B10), the cellular processing of lispro is essentially identical with that of human insulin (19). Therefore, unlike Asp(B10), lispro was found to be safe for clinical use.

In terms of activity on lipogenesis, insulin lispro was found to be essentially the same as human insulin (20). Pharmacokinetic studies indicate that insulin lispro acts within 15 min, peaks in approximately 1 h, and disappears within 2–4 h after sc injection (25, 28). In clinical studies, as expected from a short-acting analog, insulin lispro achieved significant improvements in postprandial glucose levels with a lower rate of hypoglycemic events compared with regular insulin (29–31). This can be observed even if insulin lispro is administered immediately before meals and regular insulin is injected 30–45 min before meals. Unfortunately, in most cases, these beneficial effects were not accompanied by improvements in glycosylated hemoglobin values (29, 30). In addition to the decrease in hypoglycemic events, the most likely explanation for this is the inability of the currently used long-acting insulins to provide true basal coverage. Therefore, increased preprandial plasma glucose concentrations are present in patients on insulin lispro. Supporting this theory, a clinically and statistically significant decrease of hemoglobin A_{1c} levels was seen when insulin lispro was used with two or more daily injections, instead of one, of neutral protamine Hagedorn (NPH) insulin (32, 33). Therefore, for the intensive therapy of diabetes by multiple daily injections, the addition of a few units of NPH to lispro at each meal, combined with bedtime NPH, can be recommended (33–35). This regimen may even improve unawareness of, and impaired counterregulation to, hypoglycemia (35).

Similarly, because continuous sc insulin infusion (CSII) systems are able to provide a reasonable basal insulin substitution, improved glycosylated hemoglobin values would be expected with pump treatment using insulin lispro. After the stability of lispro in insulin pump systems had been confirmed (36), clinical trials began to assess its effectiveness in CSII treatment. As assumed, results with insulin lispro in patients receiving CSII are promising, as evidenced by lower glycosylated hemoglobin values and improved postprandial glucose levels as compared with patients receiving pump treatment with regular insulin (37, 38). Importantly, the improved glycemic control is achieved without an increase (or even with a decrease) in the number of hypoglycemic events. A potential disadvantage of using insulin lispro in pump systems as opposed to regular insulin is that, because of its more rapid disappearance, patients might be at more risk for developing ketoacidosis in the case of catheter occlusion or pump malfunction (39). This was, however, not confirmed by a recent study, in which no difference with respect to the rate of rise in plasma glucose or serum ketone levels after disrupting sc infusion was found between patients receiving CSII treatment with lispro or those receiving treatment with regular insulin (40). The frequency of catheter occlusion or other site-related problems is similar with lispro and buff-

ered regular insulin (37, 38). When comparing regimens using lispro, it was found that using lispro in CSII provides better glycemic control with lower doses of insulin than multiple daily injections of lispro and NPH (41). This, in addition to supporting the suitability of lispro in pump systems, also highlights the fact that the real advantages of a short-acting analog can be better translated into clinical benefits when they are used in a regimen with optimal basal insulin coverage (*i.e.*, insulin pumps or a truly long-acting insulin, but not NPH).

A protamine formulation of insulin lispro with prolonged action neutral protamine lispro has been developed and shown to be suitable as an intermediate-acting agent or as part of premixed preparations of lispro and neutral protamine lispro (25/75 and 50/50) (42, 43). Compared with human insulin mixtures, twice-daily administration of insulin lispro mixtures resulted in improved postprandial glycemic control, similar overall glycemic control, and less nocturnal hypoglycemia, as well as offering the convenience of dosing closer to meals (44).

Managing diabetes in patients with end-stage renal disease is often problematic, because renal failure interferes with the metabolism of glucose and insulin. Many of these diabetics have wide fluctuations in their daily blood glucose profile. The action of regular insulin may be prolonged as a consequence of the failure of renal insulin degradation, making the dose-effect profile of insulin difficult to control and making hypoglycemia more likely. There is evidence that using insulin lispro might make the calculation of insulin requirements easier and might help to avoid large fluctuations in blood glucose levels of these patients (45).

Insulin lispro has also been tested for use in pregnancy and gestational diabetes (46, 47). Compared with regular human insulin, during a meal test, areas under the curve for glucose, insulin, and C-peptide were found to be significantly lower with insulin lispro. Mean fasting and postprandial glucose concentrations and end-point HbA_{1c} levels were similar to those with regular insulin, but patients on lispro demonstrated fewer hypoglycemic episodes. No fetal or neonatal abnormalities were noted in either treatment group. Anti-insulin antibody levels were similar in the two groups, and insulin lispro was not detectable in the cord blood (46). A recent study found that, whereas no patients on insulin lispro showed any change in their retinopathy status, 14% of patients on regular insulin had worsening of retinopathy (48).

Based on the limited available data on its long-term effectiveness, it appears that insulin lispro remains effective in treating diabetic patients up to 5.4 yr of treatment (49). No differences have been reported between insulin lispro and regular insulin in the likelihood of developing allergic reactions, adverse events, or abnormal laboratory values (50). The immunogenicity of insulin lispro is similar to that of regular insulin (51). Antibodies specific against insulin lispro hardly ever develop and do not affect dose requirements (49, 52). Interestingly, there have been reports of patients in whom severe resistance to human insulin due to antibody formation was successfully overcome by switching them to insulin lispro (53, 54).

Despite the difficulties with standardizing quality-of-life assessments, the available data are surprisingly consistent

and show a greatly increased treatment satisfaction among patients receiving lispro by CSII or as multiple injections (29, 38, 55). This can improve patient motivation and compliance, which are very important components of treatment success in diabetic patients.

C. Insulin aspart [NovoLog (Novo Nordisk, Princeton, NJ)]

The next example of changing the amino acid sequence of the insulin molecule to achieve short-acting insulin analogs is insulin aspart (AspB28), in which substitution of proline with the charged aspartic acid is carried out to reduce self-association of the molecule (Fig. 1 and Table 1) (56). This analog was approved for clinical use in the United States in June of 2000. Preclinical studies of insulin aspart have demonstrated that receptor interaction kinetics with the insulin receptor and with the IGF-I receptor are essentially equivalent to those seen with human insulin (22), and an equivalent metabolic effect of insulin aspart and human insulin has been shown with iv administration (57). The potency on lipogenesis of insulin aspart is similar to that on human insulin, whereas its affinity to the IGF-I receptor is slightly lower, and thus, it does not result in greater mitogenic potency (20). When administered iv, insulin aspart shows a similar safety profile with that of human insulin (58). When further assessing its safety, it was found that insulin aspart and soluble human insulin elicit the same counterregulatory and symptomatic responses to acute hypoglycemia in patients with type 1 diabetes (59). Insulin aspart has been shown to be absorbed twice as fast as human insulin and to reach maximum concentrations twice as high, whereas its duration of action is shorter (60–62). As expected, the postprandial glucose control achieved with this analog is superior to regular human insulin, whereas their bioavailability is comparable (61). Mean postprandial glucose levels after any meal are lower, even when aspart is injected immediately before the meal and regular human insulin is administered 30 min before meals (63). These results are consistent with those reported with the other short-acting analog, lispro, but there is evidence that the improvement in postprandial control can be achieved without deterioration of late postprandial plasma glucose concentrations (64). The expectation of lower rates of hypoglycemia also seems to have been met with insulin aspart, as evidenced by a recent multicenter trial of type 1 diabetic patients, which showed more than a 50% reduction in major hypoglycemic events compared with human insulin (64). In a very interesting study with type 1 diabetics, it was found that, because of its rapid absorption, insulin aspart provided reasonable glucose control even when injected 15 min after the start of meals (65). In the same study, it was also found that after abdominal injections, aspart had a shorter duration of glucose lowering effect than after administration in the thigh or deltoid area (65). The beneficial effects of insulin aspart have also been confirmed in type 2 diabetics (66) and in a pediatric population with type 1 diabetes (67). Importantly, this analog retains its beneficial pharmacodynamic properties in a stable 30/70 premixed formulation, as it shows a significantly greater metabolic effect in the first 4 h with more rapid absorption and higher peak serum concentration than the 30/70 mixture of

human insulin (68, 69). Because of its promising characteristics, studies are presently underway to evaluate long-term metabolic control with insulin aspart.

V. Long-Acting Insulin Analogs

A number of alterations of the insulin molecule by genetic engineering are currently being tested to retard and stabilize absorption kinetics of long-acting insulin preparations. One possibility to prolong insulin action is to elevate the isoelectric point of human insulin from pH 5.4 toward neutral by developing analogs with more positively charged amino acids (70). This will make the analog less soluble at the neutral pH of the injection site, and the injection of the analog into the sc tissue will result in crystallization of the molecules, causing delayed absorption into the circulation.

A. NovoSol Basal (Novo Nordisk)

One of the first analogs developed by recombinant DNA technology based on the above therapeutic goals was NovoSol Basal (B27Arg, A21Gly, B30Thr-NH₂). As evidenced by longer half-life than that of Ultratard (Novo Nordisk) HM insulin, one of the longest acting preparations of human insulin, the task of prolonged absorption was successfully completed with this NovoSol Basal, but nearly 2 times higher doses of this analog were required to achieve compatible glucose control. Also, whereas NovoSol Basal showed less intraindividual variability in its action, the interindividual variation remained high. Therefore, and also because of its reduced bioavailability, NovoSol Basal was withdrawn from further studies (71, 72).

B. Insulin glargine [HOE 901, LANTUS (Aventis Pharmaceuticals, Parsippany, NJ)]

HOE 901 (insulin glargine, LANTUS) is a new long-acting biosynthetic human insulin analog developed by Aventis Pharmaceuticals, which was approved for use in patients with type 1 and type 2 diabetes mellitus by the United States Food and Drug Administration in April of 2000 and by the European Agency for the Evaluation of Medicinal Products in June of 2000. This analog results from elongation of the C-terminal end of the insulin B chain by two arginine residues, as well as substitution of the A21 asparagine residue with glycine (A21Gly, B31Arg, B32Arg human insulin) (Fig. 1 and Table 1). These modifications led to a shift of the isoelectric point from pH 5.4 of human insulin to 6.7, making insulin glargine less soluble at physiological pH levels. After sc injection, insulin glargine precipitates in the sc tissues, which delays its absorption and prolongs its duration of action (73). The substitution at position A21 largely increased the bioavailability of this analog, so unlike NovoSol Basal, it is suitable for clinical use (74).

With respect to insulin receptor binding, receptor autophosphorylation, phosphorylation of signaling elements, and promotion of mitogenesis in muscle cells, insulin glargine behaves like regular human insulin (23). Moreover, the growth-promoting activity of HOE 901 in muscle cells and the maximal metabolic activity of this analog are not

different from those of native human insulin, whereas its lipogenic activity is slightly lower (20, 75). However, insulin glargine's therapeutic properties and potentials are remarkable and different from human insulin. HOE 901 was shown to exert a glucose-lowering effect for 24 h after a single daily injection without a pronounced plasma peak and induced a smoother metabolic effect than NPH insulin (73, 76). Thus, HOE 901 is expected to better substitute basal insulin requirements. Moreover, although it is well known from clinical practice that the effect of NPH insulin can vary with the site of injection, it has been found that changes in the injection site do not alter the time-action profile of HOE 901 (77, 78). In one of the first small, short-term clinical studies investigating this analog in 1996, once-daily injections of HOE 901 resulted in similar glycemic control as compared with four daily injections of the same total units of NPH in type 1 diabetics (79). The characteristics of HOE 901 have been investigated in both type 1 and type 2 diabetic patients. In phase II trials conducted in Europe and the United States with type 1 diabetics, once-daily injections of HOE 901 along with premeal regular insulin achieved significantly lower fasting plasma glucose levels (80) and hemoglobin A_{1c} values compared with patients on NPH and regular insulin (81). Remarkably, the better glucose control was associated with similar or even lower incidences of hypoglycemia. Studies of type 2 diabetic subjects showed similar fasting plasma glucose values with one injection of HOE 901 compared with those found with one or two injections of NPH insulin. Again, the incidence of hypoglycemia was similar or lower among patients on HOE 901 (82–84). More recently, the findings of less frequent hypoglycemic episodes and lower fasting plasma glucose levels compared with NPH were confirmed in large, multicenter clinical trials with type 1 and type 2 diabetics in Europe and the United States (85–88). Considering that less hypoglycemia was consistently observed, these data suggest that the target fasting plasma glucose level can be lower for insulin glargine than for NPH (88). The technical difficulties with blinding the studies comparing NPH and HOE 901 should be noted, as the two preparations can be easily identified because HOE 901 is a clear solution as opposed to the cloudy solution of NPH. It might make designing blinded research studies more difficult, but in daily clinical life, it could actually be an advantage that insulin glargine is a clear solution. It has been shown that patients do not sufficiently shake suspensions like NPH insulin before administration (89). Because it is not necessary to shake HOE 901 before usage, it may have a lower intra-individual variability of its metabolic effect. In recent clinical trials, patients treated with insulin glargine had less variability of their fasting plasma glucose values than those receiving NPH (84, 90).

Insulin glargine has a greater affinity to the IGF-I receptor than human insulin (20). The observation of a progression of retinopathy in some patients with type 2 diabetes treated with insulin glargine raised concerns, partly because IGF-I has been implicated in the development of retinopathy (91). A review of the retinopathy data and the lack of optic disc swelling, which is the most common ocular side effect of treatment with IGF-I, led to the conclusion that this finding was probably not related to insulin glargine (92).

A potential problem with altering the structure of the insulin molecule is increasing the risk of antibody development and adverse reactions at the site of injection. Importantly, adverse events and injection-site reactions associated with HOE 901 were not different from those found with NPH insulin, and antibody formation was also similar with the two preparations.

C. Fatty acid-acylated insulins

Another way of prolonging insulin action is by modifying the hormone's structure to achieve binding to a serum protein. It is well known that a number of hormones bind to a specific serum-binding protein, which extends their half-life. The same can be done with insulin by coupling the insulin molecule to nonesterified fatty acids, which bind to albumin. Albumin serves as a multifunctional transport protein that binds a wide variety of endogenous substances and drugs. Albumin is present in the sc tissue fluid with a slow disappearance rate. Binding insulin to albumin can therefore retard the absorption of the molecule and prolong its action. The binding to albumin apparently involves both nonpolar and ionic interactions with the protein (93). Acylation of the insulin molecule is usually performed in the side chain of lysine at position 29 of the B chain. Such insulin analogs are currently being studied by Lilly (Indianapolis, IN) (WW99-S32) and Novo Nordisk (Copenhagen, Denmark) (NN304).

1. NN304 (*insulin detemir*). In animal studies, the time for 50% disappearance from the sc space of NN304 (LysB29-tetradecanoyl, des(B30)-insulin) was 14.3 h, significantly longer than that of NPH insulin (10.5 h) and with significantly less interanimal variation (94). In healthy volunteers, the metabolic response induced by sc injection of NN304 does not show the pronounced peak seen with NPH insulin in an identical dose. NN304 also shows a slower onset of action, as indicated by a significantly higher maximal life compared with NPH insulin (95). This analog has been found to be less effective than human insulin when given in equimolar doses to healthy volunteers (95). Insulin detemir was also found to have a lower affinity to the insulin receptor, but a prolonged receptor dissociation time compared with human insulin (20). Insulin detemir is less potent than human insulin in binding to the IGF-I receptor and stimulating lipogenesis, and unlike Asp(B10), it is less mitogenic than human insulin. Thus, the *in vitro* profile of insulin detemir did not cause any safety concerns (20). Importantly, the binding of NN304 has been shown to be independent of the binding of drugs in the two major binding pockets that are located in domains IIA and IIIA of the albumin molecule. Thus, NN304 is unlikely to be involved in clinically significant drug interactions at the albumin binding level (96).

2. WW99-S32. In a diabetic animal model, the duration of action of the other fatty acid-acylated insulin analog, WW99-S32 [Nε-palmitoyl Lys(B29)] human insulin, administered iv was nearly twice that of unmodified human insulin, and the plasma half-life was nearly 7-fold that of the unmodified protein. Administered sc, [Nε-palmitoyl Lys(B29)] human insulin had a longer duration of action, a flatter, more basal plasma insulin profile, and a lower intersubject variability of

response than the intermediate-acting insulin suspension Humulin L (Eli Lilly & Co.) (97). The combination of these attributes resulted in prolonged stabilization of fasting glucose levels in insulin-dependent animals. The binding of this analog to albumin was confirmed. In human studies with healthy volunteers, this analog showed a highly reproducible, linear pharmacokinetic profile, but showed less potency when compared with NPH (98). The latter finding was subsequently confirmed in C-peptide-negative patients (99). Based on the results with insulin acylation, derivatization with albumin-binding ligands could be generally applicable to prolong the action profile of peptide drugs (93).

VI. Remaining Tasks

A. Selective action

Insulin has a number of effects in addition to carbohydrate metabolism. Some of these effects depend on the cell or tissue type studied. Therefore, selectivity of an analog can be defined as selectivity to a certain tissue, or to a certain effect.

Insulin influences glucose metabolism by inhibiting hepatic glucose production and stimulating peripheral glucose disposal. Insulin analogs with relatively greater effect on hepatic glucose production could offer potential therapeutic benefits for selected patients. Another consideration is that the pancreas delivers insulin to the portal vein, and the liver is therefore subject to relatively high insulin concentrations compared with peripheral tissues. With sc insulin therapy, this portal/peripheral insulin gradient is lost, resulting in nonphysiological insulin distribution. The result, even in patients able to achieve near-normal HbA_{1c} levels, is multiple and profound metabolic abnormalities, including excessive glycemic fluctuations, dyslipidemia, and alterations in IGF-I and GH levels. These abnormalities have been implicated in the complications of diabetes (100, 101). Currently, insulin can only be delivered into the portal circulation by surgically implanted ip pumps, certain types of pancreatic transplantation, or islet cell transplantation (102, 103). The importance of the issue is underlined by the findings of decreased requirement for antihypertensive therapy and decreased total and free insulin and insulin antibodies in patients with surgically implanted pumps (104). Unfortunately, despite the recent promising preliminary results with islet cell transplantation using a glucocorticoid-free immunosuppressive regimen, all of the above methods have significant difficulties, which preclude their use in the majority of patients (105, 106). An alternative could be the development of insulin analogs with a greater effect on the liver than on the periphery.

1. Proinsulin. Proinsulin, the single-chain precursor of insulin, is more effective in the liver than in the periphery (107, 108). Reasons for this selectivity are not fully understood, but the increased molecular size of proinsulin compared with insulin has been proposed as a potential mechanism. Endothelial cells in peripheral tissues limit the transfer of substances from the circulation into the tissues with a rate inversely related to the molecular size of the transferred substance. However, hepatocytes are freely in contact with

all blood constituents in the hepatic sinusoids. Dose requirements for proinsulin are approximately 4-fold higher than for human insulin, and there is a possible association between its use and myocardial infarction (109, 110). Proinsulin was therefore withdrawn from clinical trials, but the recognition of its selective action has stimulated the search for analogs with greater hepatic effects relative to peripheral tissues.

2. Thyroxyl-insulin complex. Two insulin analogs with increased molecular size due to covalent dimerization have been shown to have a greater effect on hepatic glucose production than peripheral glucose disposal after iv administration (111). These dimeric analogs (N α B1, N α B'1,-suberoyl-insulin dimer, and N ϵ B29, N ϵ B'29,-suberoyl-insulin dimer) are probably not suitable for clinical use because of their relatively low potency, but they confirm the possibility that analogs with selective action due to increased molecular size might be developed. Another interesting finding is that two insulin analogs covalently linked to T₄ (N α β 1-thyroxyl-insulin and N α β 1-thyroxyl-aminohexanoyl insulin) also show greater selectivity for hepatic glucose production in dogs (112). These insulin analogs bind thyroid hormone binding proteins to form high molecular weight complexes. N α β 1 L-thyroxyl-insulin was recently found to be well tolerated and well absorbed in humans after sc injection and to show hepatoselectivity compared with NPH insulin (113). These findings provide further support to the theory that the reduced peripheral insulin-like effect could be due to reduced transcapillary access to peripheral insulin receptor sites, which results from high molecular weight.

3. Further possibilities for selective action. Another possibility for selectivity of an analog, either to a specific tissue or for a specific action (e.g., increased mitogenicity compared with metabolic effects) would be altered cellular metabolism of the analog. Reduced degradation would prolong cellular residence of the material and alter activity profile. The analog Asp(B10) is an example of this (19). The metabolic effects of insulin analogs, such as increased glucose uptake and metabolism, and the mitogenic effects generally correlate well with binding to the insulin receptor (20). However, some metabolic actions, such as inhibition of protein degradation, do not. We have recently shown that the inhibition of protein degradation in cultured cells by insulin and the analogs lispro, Asp(B10), and B4Glu,B16Gln,B17Phe insulin does not correlate to insulin receptor binding and is dependent on cell type (114). For example, Asp(B10), which shows markedly increased receptor binding compared with insulin, has a similar effect to insulin on the inhibition of protein degradation in the human hepatoma cell line HepG2. This means that relative to its receptor binding, Asp(B10) is less effective in inhibiting protein degradation than human insulin in HepG2 cells. The effects in two other cell lines are dependent on the class of proteins being investigated. Effects similar to insulin were seen on short-lived proteins, but intermediate-lived protein degradation was inhibited to a greater degree with Asp(B10) than with insulin. Further work has suggested that the action to inhibit protein degradation is more closely correlated to cellular insulin/analog processing. In fact, it has been shown that insulin inhibition of protein degradation in

isolated rat hepatocytes requires cellular insulin degradation (115, 116). For future development of specific analogs, more information is needed on properties of the insulin molecule important for different biological activities, *e.g.*, carbohydrate *vs.* fat *vs.* protein metabolic effects (117). An example for this might be insulin detemir, which was found to have less lipogenic activity than human insulin relative to its insulin receptor affinity (20).

B. Increased stability

Insulin is not a stable chemical entity. A variety of chemical changes of the primary structure affect insulin during handling, storage, and even use. Insulin decomposition is mainly due to two categories of chemical reactions: hydrolysis and intermolecular transformation leading to covalent insulin dimers. Identification of the residues undergoing chemical changes during storage allows designing insulin analogs with improved stability. The advantage of such analogs would be prolonged shelf-life and more convenient storage conditions. Improved stability is also essential for pump usage. The above discussed Asp(B10) analog has increased stability but is unfortunately not suitable for clinical use (118). Substitution of AsnB3 by Gln, and AsnA21 by Ala or Gly, results in analogs with 30 times less deamination and 10 times reduced formation of covalent dimers (14). In a very interesting recent study, it was shown that attachment of short-chain (750- and 2000-Da) methoxypoly (ethylene glycol) to the amino groups of either residue PheB1 or LysB29 of insulin's B-chain improves the conjugates' physical stability without appreciable perturbations to its tertiary structure, self-association behavior, or *in vivo* biological activity (119). However, designing and testing more analogs with increased stability still remains an important task for the future.

C. Less variability

The high intra- and interindividual variability of the response to identical insulin doses is a serious problem for patients and their clinicians as well and can hamper the achievement of reasonable glycemic control without the risk for hypoglycemic events (60). There are two explanations for the variability of insulin responsiveness. Pharmacokinetic variability can result from variations in insulin absorption, leading to different plasma concentrations of insulin after sc injection of the same doses (120, 121). Pharmacodynamic variability, on the other hand, can be caused by differences in insulin action, causing different metabolic effects by similar plasma insulin concentrations (122). In short-acting preparations, a decreased variability in serum insulin concentrations compared with regular human insulin has been shown after sc injections of the analog insulin lispro (123). Also, interindividual variability in pharmacodynamic and pharmacokinetic parameters with insulin aspart was found to be generally less than that with human insulin, whereas the intraindividual variability in these parameters was similar for the two (124). Generally, variability is even more problematic with long-acting insulin products; this is due to their insoluble nature. The long-acting preparations of human

insulin are mostly suspensions, which require shaking before use, adding another factor to variability as adequate mixing usually does not occur (89). It is therefore expected that soluble long-acting analogs will have less variability in their pharmacokinetics. The above-discussed long-acting analog NovoSol Basal shows less intraindividual variation in its pharmacokinetics than the longest-acting currently available human insulin preparation Ultratard HM (71). Nevertheless, developing insulin analogs with lower inter- and intraindividual pharmacokinetic and pharmacodynamic variability remains an important task.

D. Ultrarapid onset

Although significant improvements in postprandial plasma glucose levels can be achieved with the presently available short-acting analog insulin lispro, even when it is injected immediately before meals, there is evidence that its optimal administration would actually be 15–30 min before meals (125). When administered at least 15 min before meals, lispro achieves a greater improvement in postprandial values as opposed to being injected immediately before meals. This suggests that developing even more rapidly absorbed short-acting analogs could offer potential benefits.

E. Ultralong activity

Some insulin-requiring patients simply do not have the background or resources needed for insulin treatment. They may not have access to a refrigerator or are unable to use insulin without getting help because of disabilities. These patients could potentially use ultralong-acting analogs that could be injected once weekly or even less frequently. This type of preparation obviously would not provide good control but could offer basal coverage sufficient to prevent ketoacidosis or other acute complications. The concept may seem utopian at first, but a recent study reported that a single sc injection of a new analog, in which two 9-fluorenylmethoxycarbonyl moieties are covalently linked to the phenylalanine at position B1 and to the lysine at B29 of human insulin, normalized blood sugar levels for 2–3 d of rats with streptozotocin-induced diabetes (126). The analog itself has only 1–2% of the biological potency of insulin, but undergoes a time-dependent spontaneous conversion to fully active insulin. The conversion takes place slowly under physiological conditions, with a $t_{1/2}$ of 12 d.

F. Benefit without metabolic activity?

The insulin analog Asp(B25) practically does not bind to the insulin receptor or IGF-I receptor and has no hypoglycemic effect (17). However, this analog has been shown to prevent diabetes in an animal model of spontaneous diabetes that shares many features of human type 1 diabetes (127). The analog prevented diabetes in the animals even when it was initiated after the onset of extensive lymphocytic infiltration of the pancreatic islets. The mechanism, because it did not involve metabolic effects, appears to be immunological. Preliminary trials have suggested that treatment of high-risk prediabetic patients with human insulin can prevent the onset of diabetes, but of course, this carries the risk of hy-

poglycemia, even more so than in patients with fully developed diabetes. Several large-scale controlled trials have been organized (e.g., the Diabetes Prevention Trial 1 and the European Pediatric Prediabetes Subcutaneous Insulin Trial) to evaluate the effect of prophylactic insulin therapy in the prevention or delay of diabetes in high-risk pediatric individuals (128, 129). Although it is still unclear whether the analog Asp(B25) can be used for preventing diabetes in prediabetic children and young adults, the theory of using analogs without the potentially harmful hypoglycemic effects for diabetes prevention is certainly an interesting one.

VII. Conclusions

After more than half a century of treating diabetics with animal insulins, recombinant DNA technologies and advanced protein chemistry made human insulin preparations available in the early 1980s. As the next step, over the last decade, a number of insulin analogs were constructed and tested to further improve the therapy of diabetes (20, 130). The need for nearly optimal glucose control in diabetics to minimize complications clearly exists. Without insulin analogs, however, this can only be accomplished at the expense of an increase in hypoglycemic reactions. The Diabetes Control and Complications Trial demonstrated that a 10% improvement of glycosylated hemoglobin levels results in a 43% improvement of retinopathy, but is accompanied by an 18% increase of severe hypoglycemic episodes (5). The first clinically available insulin analog, lispro, opened new hopes by showing that improved glycemic control can be achieved without an increase in hypoglycemic events. This requires, however, optimal basal insulin replacement, either by multiple daily injections of NPH or by CSII. Evidence suggests that short-acting insulin analogs would be better matched by a true basal insulin than by the erratically absorbed and rather short-acting NPH insulin (64). Therefore, the availability of long-acting analogs raises the hope to take advantage of the true potential benefits of the currently available short-acting analog lispro, and of those still awaiting approval. The introduction of new short- and long-acting analogs and the development of analogs with increased stability, less variability, and perhaps selective action will help to develop more individualized treatment strategies targeted to specific patient characteristics and to achieve further improvements in glycemic control. Combining different insulin analogs may even help to treat the multiple metabolic abnormalities diabetics have beyond their carbohydrate metabolism.

Insulin analogs also represent a unique tool to unravel structure-function relationships in insulin biochemistry and insulin action (20). Recombinant insulin analogs have been and will be important in mapping the putative receptor binding domain(s) of the insulin molecule and elucidating the specificity of the pathways leading to the metabolic and mitogenic effects of the hormone.

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